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LETTER TO THE EDITOR

Diagnostic value of genetic mutation analysis and mutation profiling of cell-free DNA in intraocular fluid for vitreoretinal lymphoma

Dear editor,

Vitreoretinal lymphoma (VRL) is a rare intraocular malignant lymphoma affecting the vitreous and/or retina. Pathological diagnosis is challenging. Retinal biopsies have high risks of irreversible visual impairment (even blindness), which are not used in clinics for patients with residual visual function. The limited cellular yield from vitreous biopsies and the cell lysis also contribute to the low detection rates of VRL using cytopathology [1]. Therefore, less invasive intraocular fluid (IOF) sampling techniques, such as vitreous aspiration and anterior chamber paracentesis, have been used to obtain samples for diagnostic tests. However, diagnostic tests for VRL that involve the use of IOF samples, including interleukin-10 (IL-10)/interleukin-6 (IL-6) ratio and immunoglobulin gene rearrangement examinations, are also affected by ocular inflammatory conditions, which has resulted in unsatisfactory sensitivity and specificity [2]. An alternative is liquid biopsy for the detection of tumor-derived genetic alterations in cell-free DNA (cfDNA), although it has been underutilized previously. Recent studies on VRL evaluated only a small quantity of genetic alterations or a limited number of patients [3–5].

Abbreviations: VRL, Vitreoretinal lymphoma; IOF, intraocular fluid; IL-10, interleukin-10; IL-6, interleukin-6; cfDNA, cell-free DNA; NGS, next-generation sequencing; ctDNA, circulating tumor DNA; AH, aqueous humor; VF, vitreous fluid; MAF, mean mutation allele frequency; *IKZF3*, IKAROS family zinc finger 3; *ASXL1*, additional sex combs-like 1; *CHEK2*, checkpoint kinase 2; *POT1*, protection of telomeres; *FLT4*, fms related receptor tyrosine kinase 4; *PIM1*, Pro-viral integration site for moloney murine leukemia virus-1; *MyD88*, myeloid differentiation primary response protein 88; *ETV6*, Ets variant 6; *IRF4*, interferon regulatory factor 4; *DLBCL*, diffuse large B-cell lymphoma; COO, cell-of-origin; PCNSL, primary central nervous system lymphoma; MCD, *MyD88*/*CD79B*-mutated; *Bcl-6*, B-cell lymphoma 6; *NOTCH2*, Notch receptor 2; *BN2*, Bcl-6/NOTCH2-mutated; *BTG2*, B-cell translocation gene 2; *MYC*, Myc Proto-Oncogene; *CREBBP*, cAMP-response element binding protein; *FAT4*, FAT atypical cadherin; *MED12*, mediator complex subunit.

In the present study, we analyzed the genetic mutation profiles of patients with a known diagnosis of VRL or uveitis. Next-generation sequencing (NGS) with a panel containing 446 tumor-related genes (Supplementary Table S1) was performed to investigate the diagnostic value of this panel for VRL and to exhibit the genetic mutation profile of VRL. We compared the presence of circulating tumor DNA (ctDNA) in patients with VRL and uveitis in both training and validation cohorts (Supplementary Tables S2-S3).

The training cohort consisted of 17 patients with VRL and 6 with uveitis diagnosed at Zhongshan Ophthalmic Center of Sun Yat-sen University (Guangzhou, Guangdong, China) between April 1, 2018, and March 1, 2021. IOF samples, including aqueous humor (AH) and vitreous fluid (VF) samples, were collected before treatment (Supplementary Methods). Paired samples of AH and VF were collected simultaneously from the same eyes of 6 patients with VRL and 1 with uveitis. When this was not possible, unpaired samples (10 AH and 6 VF samples) were collected from 11 patients with VRL and 5 with uveitis.

In the training cohort, all 17 patients with VRL were ctDNA-positive, whereas only 2 of 6 patients with uveitis were ctDNA-positive (Supplementary Figure S1, Supplementary Table S4). The cfDNA concentrations in AH and VF samples did not differ significantly between patients with VRL and uveitis, whereas the ctDNA concentration, mean mutation allele frequency (MAF), and number of somatic mutations were significantly higher in patients with VRL than in patients with uveitis in both AH (Figure 1A) and VF samples (Figure 1B).

The genetic mutation analysis for VRL diagnosis resulted in a sensitivity of 100%, a specificity of 66.7%, positive and negative predictive values of 89.5% and 100%, and a test efficiency of 91.3% (Supplementary Table S5). Some clonal hematopoietic mutations were detected in the patients with uveitis (Supplementary Table S6), which increased the false positive rate of our panel.

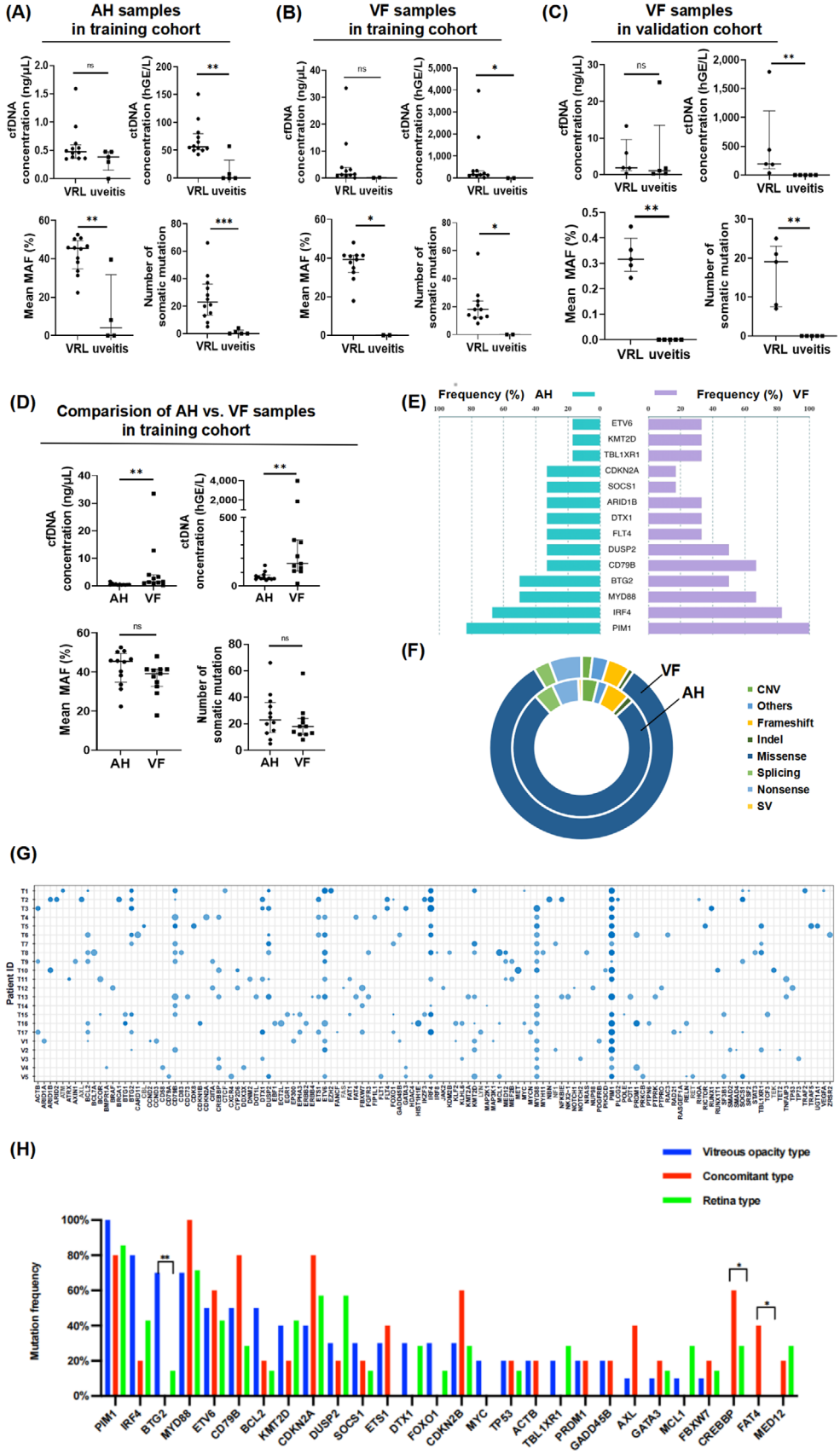

FIGURE 1 Genetic mutations in intraocular fluid (IOF) samples (i.e., aqueous humor [AH] and vitreous fluid [VF] samples) from patients with vitreoretinal lymphoma (VRL) or uveitis. (A, B) Genetic mutations in AH and VF samples from patients with VRL or uveitis in the training cohort. (C) Genetic mutations in VF samples from patients with VRL or uveitis in the validation cohort. (D) Comparison of

To validate our findings, 5 patients with VRL and 5 patients with uveitis diagnosed at Beijing Chaoyang Hospital (Beijing, China) between April 1, 2018 and March 1, 2021 were enrolled into the validation cohort (Supplementary Methods). VF samples from these patients were sent for single-blind genetic mutation analysis. The 5 patients with VRL were ctDNA-positive, whereas the 5 patients with uveitis were ctDNA-negative (Supplementary Figure S1). Although there were no significant differences in cfDNA concentration between patients with VRL and uveitis, patients with VRL did have significantly higher ctDNA concentration, MAF, and number of somatic mutations than patients with uveitis (Figure 1C, Supplementary Table S4). The sensitivity and specificity of this genetic mutation analysis in the validation cohort for the diagnosis of VRL were both 100%.

We also compared genetic mutations between AH and VF samples from patients with VRL in the training cohort to evaluate the relative diagnostic value of these two IOF sample types. The cfDNA and ctDNA concentrations were significantly higher in VF samples than in AH samples. However, MAF and the number of somatic mutations did not differ between them (Figure 1D). Furthermore, the frequencies of the top 12 high-frequency mutated genes and mutation types were similar in paired AH and VF samples (Figure 1E-F). In addition, among the paired IOF samples from 6 patients with VRL, the ctDNA positive rates were consistent. These findings suggest that AH and VF samples have similar diagnostic potential for VRL. However, considering the relative safety and simplicity of anterior chamber paracentesis, AH sampling may be more practical than VF sampling for liquid biopsy and genetic mutation analysis.

Information about the molecular features of lymphoma may be used to guide treatment and predict prognosis. Thus, we analyzed the mutation profiles and identified the molecular subtypes of the 22 patients with VRL (Supplementary Table S6). The 5 most frequently mutated genes found in these patients were proviral integration site for moloney murine leukemia virus-1 (*PIM1*) in 90.9%; myeloid differentiation primary response protein 88 (*MyD88*) in 77.3%; *CD79B* in 50.0%; ETS variant 6 (*ETV6*) in 50.0%; and interferon regulatory factor 4 (*IRF4*) in 50.0% (Figure 1G). More *PIM1* and *IRF4* mutations, but fewer *MyD88* mutation were observed in VRL patients than primary central nervous system lymphomas (PCNSL) patients as we previously reported [6]. Although two patients with uveitis were ctDNA-positive, their ctDNA concentrations were low; the observed mutations in the two patients with ctDNA-positive uveitis are uncommon in B-cell lymphomas, and we considered them to be clonal hematopoietic mutations (Supplementary Table S6).

Among the 22 patients with VRL, 10 presented as vitreous opacity type, 7 presented as retina type, and 5 presented as concomitant type. We compared the mutation frequencies in these types and observed that they were different (Figure 1H).

Molecular classification of diffuse large B cell lymphoma (DLBCL) has become increasingly important because of its prognostic significance and the development of subtype-specific therapeutics. We were able to classify our 22 patients with VRL as non-germinal center B-cell-like (21 patients, 95.5%) and unclassified subtypes (1 patient, 4.5%) based on the cell-of-origin (COO) classification system described by Scherer et al. [7]. Recently, Wright et al. [8] described a LymphGen-based probabilistic algorithm to facilitate the application of DLBCL genetic subtyping, which was associated with the response to immunochemotherapy. We determined the genetic subtype and the predictor score of each VRL patient according to the algorithm (Supplementary Table S7). This classification of genetic subtype applied to 18 (81.8%) of our patients with VRL, of which 17 were classified as the *MyD88/CD79B*-mutated (MCD) subtype and 1 was classified as the B cell lymphoma 6 (*Bcl-6*)/Notch receptor 2 (*NOTCH2*)-mutated (BN2) subtype. Patient T17, the single unclassified case based on the COO classification, was classified as BN2 subtype by the LymphGen algorithm with a 0.76 confidence. The high frequency of MCD subtype in our cohort was consistent with a previous report that PCNSL represents MCD genetic subtype of DLBCLs [9], and MCD gene expression was enriched in immune-privileged sites (e.g., 48%-56% of PCNSL) [10].

In conclusion, cfDNA genetic mutation analysis of IOF samples was feasible and had 100% sensitivity for the diagnosis of VRL. The 446-gene panel we used allowed for genetic profile tracking, revealing the genetic

genetic mutations between paired AH and VF samples from patients with VRL in the training cohort. (E, F) Comparison of frequencies of top 12 mutated genes and mutation types between paired VF and AH samples from patients with VRL in the training cohort. (G) Bubble plot of all detectable gene mutations in the 22 patients with VRL. MAF is represented by the size of bubbles. (H) Mutation type-stratified frequencies of genes in the 22 patients with VRL. MAF for *BTG2* was significantly higher in vitreous opacity type than retinal type; *MYC* was only detected in vitreous opacity type; *CREBBP*, *FAT4*, *MED12* were not detected in vitreous opacity type. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
Abbreviations: MAF, mutation allele frequency; *BTG2*, B-cell translocation gene 2; *MYC*, Myc Proto-Oncogene; *CREBBP*, cAMP-response element binding protein; *FAT4*, FAT atypical cadherin; *MED12*, mediator complex subunit.

heterogeneity and molecular characteristics of patients with VRL.

DECLARATIONS

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CONFLICT OF INTERESTS

Xiaoyu Hong and Chuqiao Liang are employee of Nanjing Geneseeq Technology Inc., Zhuyun Qian and Ziqiang Li are employee of Beijing GIANTMED Medical Diagnostics Lab. This work had no financial support from these two companies. The other authors declare no potential conflicts of interest.

AUTHORS' CONTRIBUTIONS

YH, XC, DL, HH,and YT were responsible for the study design, data collection, data analysis, and manuscript writing. XC, YH, WS, SY, XW, PZ, ZQ, HH, YT, and DL contributed to the patients' follow-up and clinical data collection. WS, YT, HH, and DL provided guidance throughout the study. XW, PZ, XH, CL, HH, and YT helped conducting the experiment. XH, CL, ZQ, and ZL helped during data analysis and manuscript writing. HH, YT, and DL were responsible for the revision of the manuscript, and final manuscript approval. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT

The human sequence data generated in this study are not publicly available due to patient privacy requirements but are available from the corresponding authors upon reasonable request. All other data generated in this study are available within the article and its supplementary documentation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

A written informed consent was collected from each patient and the study design was approved by the Ethics Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (NO.2021KYPJ190). All procedures were performed in compliance with the principles of the Helsinki Declaration.

CONSENT FOR PUBLICATION

Not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.